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[7] Application of Random Peptide Phage Display to the Study of Nuclear Hormone Receptors

By CHING-YI CHANG, JOHN D. NORRIS, MICHELLE JANSEN, HUEY-JING HUANG, and DONALD P. MCDONNELL

Introduction

The steroid–nuclear hormone receptors are ligand-activated transcription factors involved in the regulation of a variety of processes ranging from reproduction to cholesterol metabolism. One of the research focuses in our laboratory has been to understand the mechanisms underlying the pharmacology of estrogen receptor (ER) ligands. The classical models of ER pharmacology held that the receptor exists in either an active or an inactive state within target cells. According to this model, the function of an agonist is to transform a receptor from an inactive one to an active one. Antagonists, on the other hand, were believed to competitively inhibit estrogen binding and freeze the receptor in an inactive state. This simple model was challenged when it was determined that tamoxifen, an antiestrogen used to oppose estrogen action in ER-positive breast tumors, could function as an agonist in some tissues including the bone, uterus and the cardiovascular system. Another compound, raloxifene, has been shown to function as an antagonist in the breast and uterus, while functioning as an estrogen in the bone and cardiovascular system. Reflecting these properties, tamoxifen, raloxifene, and other mechanistically similar compounds have been reclassified as Selective Estrogen Receptor Modulators (SERMs). It has been difficult to reconcile SERM action with the classical models of receptor pharmacology. However, a more complex model which more adequately describes these results has recently emerged. This is based on observations that the shape of ER is regulated by the nature of the bound

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ligand and the ability of cells to distinguish between different receptor conformations. Specifically, it was demonstrated that the ER can adopt multiple conformations upon binding different ligands.[1,2] The impact of such conformational changes was further revealed when steroid receptor coactivator-1 (SRC-1), and subsequently other cofactor proteins, coactivators and corepressors, were isolated.[3,4] The cue for recruitment of coactivators and corepressors to the ER is provided by the conformational changes induced by ligand binding to the receptor. Analysis of the crystal structure of the ER ligand-binding domain revealed that the activation function 2 (AF-2) pocket, when bound by an agonist, undergoes a conformational change which allows the docking of a conserved leucine-rich LxxLL (L = leucine, x = any amino acid) motif present in all p160 coactivator proteins. Binding of an antagonist, conversely, alters the AF-2 structure so that it is incompatible with coactivator docking.[5,6] It is thus believed that the pharmacology of a SERM is determined by both the receptor conformation it induces, and the relative expression levels of cofactor proteins in the target tissue. Therefore, it was clear several years ago that defining the impact of conformation on receptor–cofactor interactions would be instructive in understanding SERM action.

In pursuing the concept that protein–protein interactions are important determinants of nuclear receptor transcriptional activity and that such interactions are dictated by receptor conformation, many investigators have used the yeast two-hybrid screen, expression cloning, proteomic analysis, crystallography, and various biochemical methods to identify receptor-interacting proteins and to define cofactor–receptor interfaces. To complement these other studies, we have applied a random peptide phage display approach to define and determine the protein–protein interaction surfaces on the ER. Phage display has been widely used as a method to map protein–protein interactions, identify peptide ligands for cell surface receptors, and map antibody–antigen epitopes.[7] Using phage display,

[1] J. M. Beekman, G. F. Allan, S. Y. Tsai, M.-J. Tsai, and B. W. O'Malley, *Mol. Endocrinol.* **7**, 1266 (1993).

[2] D. P. McDonnell, D. L. Clemm, T. Hermann, M. E. Goldman, and J. W. Pike, *Mol. Endocrinol.* **9**, 659 (1995).

[3] N. J. McKenna and B. W. O'Malley, *Nat. Med.* **6**, 960 (2000).

[4] N. J. McKenna, R. B. Lanz, and B. W. O'Malley, *Endocr. Rev.* **20**, 321 (1999).

[5] A. K. Shiau, D. Barstad, P. M. Loria, L. Cheng, P. J. Kushner, D. A. Agard, and G. L. Greene, *Cell* **95**, 927 (1998).

[6] A. C. Pike, A. M. Brzozowski, R. E. Hubbard, T. Bonn, A. G. Thorsell, O. Engstrom, J. Ljunggren, J. A. Gustafsson, and M. Carlquist, *EMBO J.* **18**, 4608 (1999).

[7] R. Cortese, P. Monaci, A. Luzzago, C. Santini, F. Bartoli, I. Cortese, P. Fortugno, G. Galfre, A. Nicosia, and F. Felici, *Curr. Opin. Biotechnol.* **7**, 616 (1996).

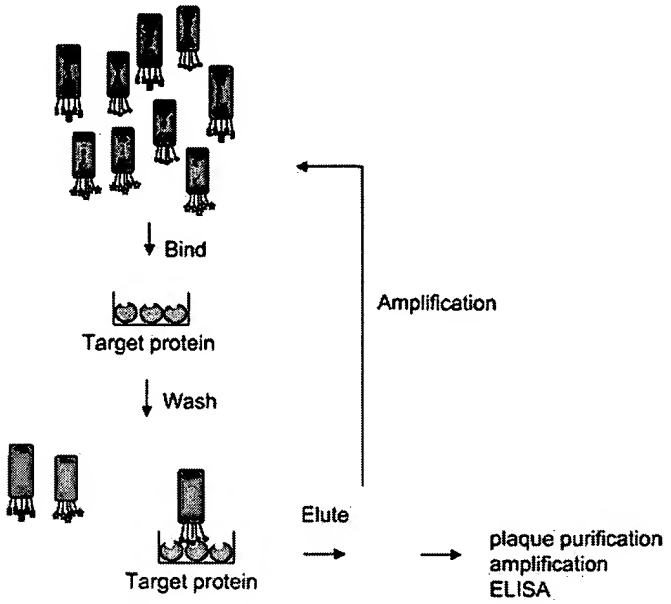

FIG. 1. Affinity selection of target protein binding peptides using phage display. M13 phage-based random peptide libraries are incubated with target protein immobilized on a solid support. After incubation, a washing step is used to remove unbound phage and the target protein binding phage are retained. Bound phage are eluted using a low-pH buffer, amplified in bacteria and subjected to subsequent rounds of selection. The selection process is repeated 3–4 times to enrich for target binding phage. Individual phage are then plaque purified, amplified, and their binding characteristics examined by ELISA. Phage that interact specifically with target proteins are selected and the sequences of the displayed peptides are deduced by DNA sequencing.

random peptide libraries can be generated and specific target-interacting peptides can be identified. The random peptide libraries are created by inserting short random oligonucleotides within the coding sequence of the M13 bacteriophage capsid protein, with the subsequent display of random peptides on the outer surface of the phage. Using routine molecular biology techniques, phage libraries containing billions of random peptide inserts can be easily constructed for use in affinity selection for target protein binders. Since the peptide is physically linked to the phage particle, target-binding phage can be recovered and amplified after affinity selection, and the oligonucleotide insert sequence can be determined by DNA sequencing to identify the corresponding peptide sequence (Fig. 1). Consequently, only a modest amount of time, effort and material are required to survey a vast number of peptides for their ability to bind to the target protein of interest.

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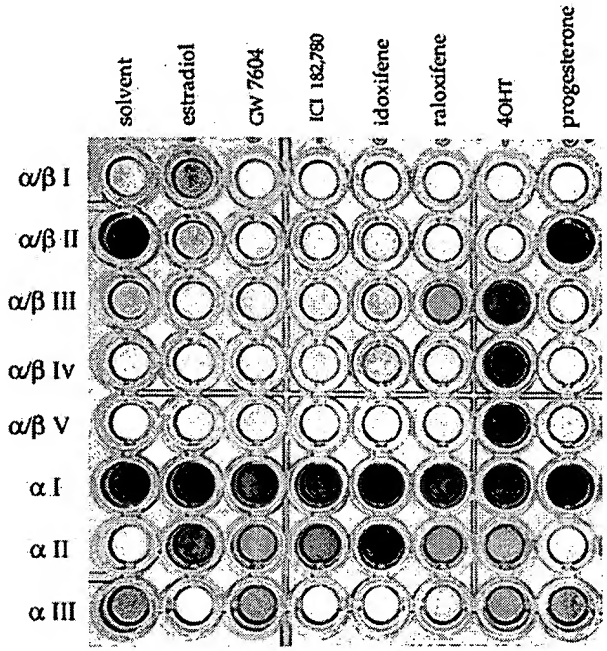

FIG. 2. Effect of ligands on ERα conformation. Biotinylated vitellogenin EREs (2 pmol) were immobilized on a 96-well plate coated with streptavidin. Subsequently, ERα (3 pmol) was immobilized on the ERE and incubated with ligand (1 μM) for 5 min before the addition of phage expressed peptides. Phage were incubated for 30 min at room temperature, and washed five times to remove unbound phage. The bound phage were detected using an anti-M13 antibody coupled to HRP and developed in ABTS and hydrogen peroxide. [Reprinted with permission from A. L. Wijayaratne, S. C. Nagel, L. A. Paige, D. J. Christensen, J. D. Norris, D. M. Fowlkes, and D. P. McDonnell, Comparative analyses of the mechanistic differences among antiestrogens. *Endocrinology* **140**, 5828–5840 (1999). Copyright The Endocrine Society.]

We have successfully used phage display to probe the various conformations of ER induced by binding to different SERMs (Fig. 2).[8,9] Characterization of ligand-induced conformational changes serves three purposes: (A) it enables the establishment of a link between the receptor conformation and biological activity, (B) it will lead to the identification of specific protein–protein interaction surfaces which may be important for receptor function, and (C) it allows the identification of novel ligands with altered peptide-binding specificities–biological functions. For example, one

[8] A. L. Wijayaratne, S. C. Nagel, L. A. Paige, D. J. Christensen, J. D. Norris, D. M. Fowlkes, and D. P. McDonnell, *Endocrinology* **140**, 5828 (1999).

[9] J. D. Norris, L. A. Paige, D. J. Christensen, C.-Y. Chang, M. R. Huacani, D. Fan, P. T. Hamilton, D. M. Fowlkes, and D. P. McDonnell, *Science* **285**, 744 (1999).

can imagine that a common biological response shared by all ER ligands is expected to result from the interaction of a common cofactor with a surface on ER that is formed regardless of which compound is used as an activating ligand (for instance, the αII peptide-binding site). On the other hand, a unique conformation created on the surface of tamoxifen-activated ER (for example the α/β V peptide-binding site) may be the surface used by tamoxifen–ER complex to recruit a novel cofactor that is specific to only this receptor–ligand complex, and which may be responsible for the partial agonist activity manifest by tamoxifen. This hypothesis was confirmed by the demonstration that the α/β V peptide was able to disrupt tamoxifen-induced ERα transcriptional activity but had no effect on the activity induced by estradiol, suggesting that the mechanism by which tamoxifen and estradiol manifest their activity may not be the same (Fig. 3). Regardless, by defining these receptor–cofactor interaction surfaces, it now appears possible to predict the biological activity of an unknown compound based on the conformation it induces within the receptor. Furthermore, since the interaction between receptor and cofactor ultimately determines the biological output of a compound–receptor complex, one should be able to generate novel classes of antagonists which target specific protein–protein interactions.

In addition to using phage display to study receptor conformation and SERM pharmacology, we have used this powerful technique to develop highly specific peptide antagonists which block receptor function by interfering with required protein–protein interactions. This approach has been particularly useful in the studies of ERα and ERβ where specific small molecule modulators are not yet available. We were able to find a small peptide that binds to ERβ but not ERα using phage display. Importantly, when introduced into cells this peptide specifically disrupted ERβ transcriptional activity without affecting the activity of the ERα protein (Fig. 4). In this post-genome era, it is also possible to use peptide sequences obtained from phage display screens to identify *bona fide* cofactor proteins and define unexpected interactions between known proteins by sequence analysis.[10,11] It is worth emphasizing that the phage display of random peptides is a powerful technology which clearly has utility in the study of most protein–protein interactions.

Although the *in vitro* phage display screen approach allows for the identification of small peptides that can probe the surface of the receptor, the biological significance of these surfaces cannot be inferred

[10] X. Li, E. A. Kimbrel, D. J. Kenan, and D. P. McDonnell, *Mol. Endocrinol.* **16**, 1482 (2002).
[11] X. Li and D. P. McDonnell, *Mol. Cell. Biol.* **22**, 3663 (2002).

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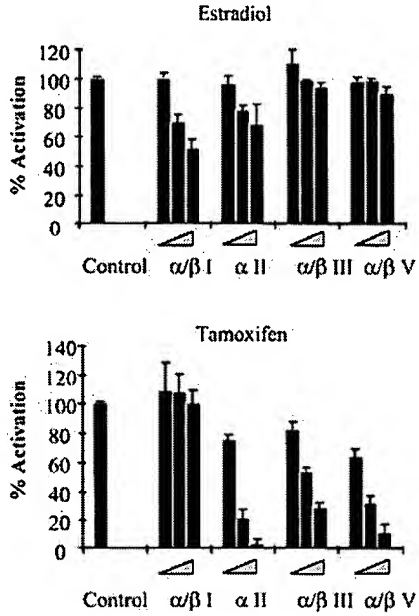

FIG. 3. Disruption of ERα transcriptional activity using cofactor-specific peptides. Cofactor-specific peptides can be used to selectively disrupt estradiol- or tamoxifen-activated ERα transcriptional activity. HepG2 cells were transfected with the estrogen-responsive C3-Luc reporter gene along with an ERα expression vector. Also included in the transfections were increasing amounts of different peptide expression vectors (as Gal4DBD fusions) as indicated. Cells were treated with 10 n*M* estradiol or 10 n*M* 4-hydroxytamoxifen. Control represents the transcriptional activity of estradiol-activated ER (or 4-hydroxytamoxifen-activated ER) in the presence of the Gal4DBD alone and is set at 100% activity. Increasing amounts of each Gal4DBD-peptide fusion expression vectors were introduced into cells (triangle) and the resulting transcriptional activity presented as percent activation of control is shown. The estradiol–ER binding peptide, *α/β* I, specifically blocked the interaction between ER and coactivator(s) recruited by the estradiol–ER complex leading to disruption of estradiol-induced ER transcriptional activity without affecting the activity induced by tamoxifen. The reverse is also true for tamoxifen–ER binding phage, *α/β* III and *α/β* V. Thus tamoxifen and estradiol do not manifest activity in the same manner. [Reprinted with permission from J. D. Norris, L. A. Paige, D. J. Christensen, C.-Y. Chang, M. R. Huacani, D. Fan, P. T. Hamilton, D. M. Fowlkes, and D. P. McDonnell, Peptide antagonists of the human ER. *Science* **285**, 744–746 (1999). Copyright 1999 American Association for the Advancement of Science.]

based solely on *in vitro* binding studies. A cell-based analysis is therefore required, and has been implemented to complement the *in vitro* approach. The cell-based approach is the modification of a commonly used mammalian two-hybrid assay to assess the interaction between the isolated peptides and the receptor(s) of interest inside cells and will be discussed later in this chapter.

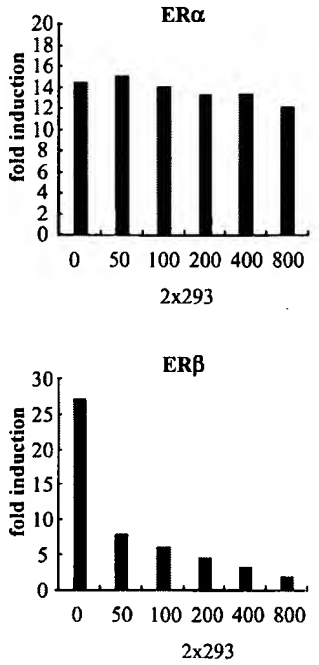

FIG. 4. An ER subtype-specific peptide selectively disrupts ERβ-dependent reporter gene expression without affecting ERα-mediated transcription when expressed in target cells. HeLa cells were transfected with an ER-responsive 3xERE-TATA-luc reporter gene alone with either an ERα or ERβ expression plasmid. Increasing amounts of the ERβ-selective peptide #293 (as Gal4DBD fusion) was also included in the transfection as indicated. Fold induction represents the ratio of the activity induced by estradiol versus no-hormone control for each transfection. [Reprinted with permission from C.-Y. Chang, J. D. Norris, H. Grøn, L. A. Paige, P. T. Hamilton, D. J. Kenan, D. M. Fowlkes, and D. P. McDonnell, Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of ERs alpha and beta. *Mol. Cell. Biol.* **19**, 8226–8239 (1999). Copyright 1999 American Society for Microbiology.]

This article is organized into four sections: the construction of a peptide phage display library, a step-by-step protocol for biopanning (the affinity selection process), the discussion of a mammalian cell-based screening technology to validate and complement the *in vitro* phage display approach, and finally the use of phage display selected peptides as antagonists of nuclear receptor function.

Construction of Random Peptide Libraries in Bacteriophage M13

Several types of bacteriophage and different coat proteins have been used to construct random peptide libraries, among which the filamentous

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M13 phage and its capsid protein pIII are the most frequently used. Due to space limitations, we will discuss only the construction and biopanning of this type of random peptide library. A detailed discussion of other library formats can be found elsewhere.[12]

Several random peptide libraries are available commercially through New England Biolabs (Beverley, MA). However, they exist in limited formats and for most applications, the investigators will have to generate their own libraries. Construction of the random peptide library involves three steps: (1) generation of double stranded oligoucleotides, (2) ligation of oligonucleotides into the M13 phage genome, and (3) transformation of ligated DNA into *Escherichia coli* cells.

Materials

All the standard buffers and solutions can be found in Current Protocols in Molecular Biology.[13]

Common chemicals are obtained from Sigma-Aldrich (http://www.sigma-aldrich.com).

SDS-PAGE purified, degenerate oligonucleotides (Life Technologies, http://www.lifetech.com).

*Xho*I, *Xba*I and Klenow polymerase (Boehringer Mannheim Corp., Indianapolis, IN).

T4 DNA ligase (New England Biolabs, http://www.neb.com).

mBAX vector (a gift from Dr. Daniel Kenan, Duke University, Durham, NC). A similar vector is available from New England Biolabs.

15% nondenaturing polyacrylamide gel:

5 ml 30% acrylamide solution (29:1) (Bio-Rad Laboratory, Hercules, CA)
1 ml 10 × TBE
4 ml H_2O
100 μl 10% ammonium persulfate
10 μl *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED)

Phenol–chloroform–isoamyl alcohol (25:24:1) (Life Technologies, http://www.lifetech.com).

3 *M* sodium acetate (pH 5.2)
Dissolve 408 g sodium acetate · $3H_2O$ in 800 ml H_2O.
Add H_2O to 1 liter.
Adjust pH to 5.2 with acetic acid.

[12] B. K. Kay, J. Winter, and J. McCafferty, "Phage Display of Peptides and Proteins: A Laboratory Manual," p. xxii. Academic Press, San Diego, 1996.

[13] F. M. Ausubel, "Current Protocols in Molecular Biology." John Wiley, New York, 2001.

2xYT medium
Dissolve 31 g 2xYT broth (Life Technologies) in 900 ml H_2O.
Adjust volume to 1 liter with H_2O.
Sterilize by autoclaving.

2xYT plates
Dissolve 31 g 2xYT broth (Life Technologies) in 900 ml H_2O.
Add 15 g bacto-agar (Life Technologies), adjust volume to 1 liter with H_2O.
Sterilize by autoclaving.
Let the agar cool to around 55 or 56°C before pouring plates.

Top Agar
Dissolve 31 g 2xYT broth (Life Technologies) in 900 ml H_2O.
Add 8 g bacto-agar (Life Technologies), adjust volume to 1 liter with H_2O.
Sterilize by autoclaving.

SOC (Life Technologies)
30% (w/v) PEG 8000–1.6 *M* NaCl
Dissolve 300 g of PEG 8000 and 93.6 g NaCl in H_2O to a final volume of 1 liter. Filter sterilize.

Methods

Generation of Double-Stranded DNA Inserts

Oligonucleotides can be custom synthesized by any vendor; however, the codon schemes used to generate random peptide inserts need to be carefully considered before placing the order. For example, NNN, where N is an equimolar mix of all four bases, will produce all four possible codons that encode all 20 amino acids. Unfortunately, this codon scheme also encodes the three stop codons, leading to generation of non-productive clones containing pre-matured stop codons. The use of either the NNK (K = G or T) or the NNS (S = G or C) codon scheme is a partial solution to this problem, because both of them use 32 codons to encode all 20 amino acids and one stop codon (TAG), significantly reducing the occurrence of a stop codon in the insert. Other alternative synthesis approaches have been used to encode all the amino acids with no stop codon, but they require more sophisticated and expensive technologies.[14–16]

[14] S. M. Glaser, D. E. Yelton, and W. D. Huse, *J. Immunol.* **149**, 3903 (1992).
[15] J. Sondek and D. Shortle, *Proc. Natl. Acad. Sci. USA* **89**, 3581 (1992).
[16] B. P. Cormack and K. Struhl, *Cell* **69**, 685 (1992).

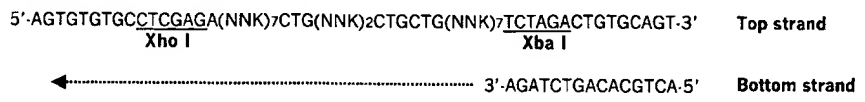

FIG. 5. The design of the oligonucleotides used in the construction of the "LxxLL" library. The top strand oligonucleotide was designed to contain an *Xho*I site (CTCGAG) at the 5'-end and an *Xba*I site (TCTAGA) at the 3'-end, which are compatible with the cloning site in the M13 phage vector mBAX and will generate inframe fusions with the pIII capsid protein. A shorter oligonucleotide complementary to the 3' sequences of the top strand is synthesized and annealed to the top strand oligonucleotide. The resulting DNA complex is extended with Klenow polymerase to generate double stranded DNA and subsequently digested with *Xho*I and *Xba*I to ligate into mBAX vector, also digested with the same enzymes. N: any nucleotide, K: G or T.

For instructional purposes, we will discuss the construction of a library in the format of -$(X)_7$LXXLL$(X)_7$- (X = A, C, G, or T) using the NNK codon scheme.

The top strand oligonucleotide (Fig. 5) was designed to contain an *Xho*I site (CTCGAG) at the 5'-end and an *Xba*I site (TCTAGA) at the 3'-end, which are compatible with the cloning site in the M13 phage vector mBAX and will generate inframe fusions with the pIII capsid protein. A shorter oligonucleotide complementary to the 3' sequences of the top strand was synthesized and annealed to the top strand oligonucleotide. The resulting DNA complex was extended with Klenow polymerase to generate double stranded DNA and subsequently digested with *Xho*I and *Xba*I to ligate into mBAX vector, also digested with the same enzymes. The ligated DNA was electroporated into *E. coli* JS-5 cells to generate phage libraries.

1. Combine 400 pmol top strand oligonucleotide
   400 pmol bottom strand oligonucleotide
   20 μl 10 × Klenow buffer (final = 1 ×)
   Adjust with H_2O to final volume of 200 μl
2. Incubate the mixture at 75°C for 15 min. Allow the reaction to cool slowly to room temperature.
3. Add
   4.0 μl 10 m*M* dNTPs (final = 200 μM)
   2.0 μl acetylated BSA (10 mg/ml) (final = 0.1 μg/μl)
   2.0 μl 100 m*M* DTT (final = 1 m*M*)
   8.0 μl Klenow polymerase (2 unit/ul)
   Incubate at 37°C for 1 hr.
4. Heat inactivate Klenow polymerase at 65°C for 1 hr.
5. Combine the double stranded DNA with
   40 μl 10 × restriction enzyme buffer (final = 1 ×)
   4 μl acetylated BSA (10 mg/ml) (final = 0.1 μg/μl)

4 μl 100 m*M* DTT (final = 1 m*M*)
Adjust with H_2O to final volume of 400 μl

Save 10 μl as no-enzyme control. Split the remaining mix into two tubes. To one tube add 100 units of *Xho*I and to the other 100 units of *Xba*I. Save 10 μl from each single enzyme digest and combine the remaining solutions into one tube to generate double digested insert. Incubate at 37°C for 3 hr.

6. Load the no-enzyme control, single-enzyme digested and double-digested samples on a preparative 15% nondenaturing polyacrylamide gel. Run the gel at 100 V until the bromophenol blue dye reaches the bottom. After electrophoresis, stain the gel with 0.2 μg/ml ethidium bromide and visualize under a UV light box to determine if the enzyme digest is complete.
7. Upon completion of the enzyme digestion, load the double digested DNA onto a 15 cm × 15 cm × 1 mm 15% polyacrylamide gel to separate any undigested or single-cut DNA from double digested DNA. Stain the gel as described above and use a razor blade to isolate gel pieces containing the double digested DNA. Collect all the gel pieces in 2–3 microcentrifuge tubes.
8. Recover the DNA insert by crushing the gel slices against the wall of the microcentrifuge tube using a pipet tip. Elute the DNA by adding 3.0 ml of 0.5 *M* ammonium acetate and incubate the tube at 37°C overnight with end-to-end rocking. Pellet the gel pieces by centrifugation. Save the supernatant, which contains eluted DNA. Reduce the volume to less than 500 μl by repeated extraction with 1 volume of 1-butanol. The DNA should remain in the aqueous phase (bottom layer).
9. Extract the eluted DNA solution with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1). The DNA should stay in the aqueous phase (top layer). Precipitate the extracted DNA with 0.1 volume of 3 *M* sodium acetate (pH 5.2) and 2.5 volume of ice-cold 100% ethanol. Pellet the DNA by centrifugation at 14,000 rpm for 20 min. Discard the ethanol and wash the pellet with 0.5 ml ice-cold 80% ethanol. Air dry the pellet and dissolve the DNA in 200 μl TE buffer. The DNA can be stored at −80°C or taken to the ligation step.

Preparation of Vector

Several M13 vectors have been constructed to accept insert DNA for phage display. The vector we use (mBAX) contains a TAG stop codon within its parental insert. The parent vector can only be propagated

in a bacterial strain that carries suppressor tRNAs (*Sup*E or *Sup*F), such as DH5αF′ or TG-1, but not a strain that lacks suppressor tRNA, such as JS5. Libraries constructed with this vector will eliminate the production of non-recombinant phage and select for only phage containing the inserts when propagated in JS-5 cells. Detailed descriptions of other types of M13 cloning vectors can be found elsewhere.[12]

1. Combine 250 μg mBAX DNA
   100 μl restriction enzyme buffer
   10 μl acetylated BSA (10 mg/ml)
   10 μl 100 m*M* DTT
   Adjust with H_2O to final volume of 1000 μl.
2. Add 300 units of *Xho*I and 300 units of *Xba*I as described in preparation of inserts. Incubate at 37°C for 3 hr.
3. Check the completeness of digestion by running samples on an 0.8% agarose gel.
4. Extract and precipitate DNA as described in the preparation of insert DNA.
5. Dissolve the vector DNA in 500 μl TE buffer.

Ligation of Insert and Vector DNA

1. In a microcentrifuge tube,
   combine 100 μg vector DNA
   appropriate amount of insert DNA
   400 μl of 10 × T4 DNA ligase buffer
   25 μl of T4 DNA ligase (125 Weiss unit total)
   Adjust with H_2O to final volume of 4000 μl.
   Incubate at 15°C overnight.
   Note. The amount of insert DNA to be used in the ligation should be pre-determined in a small-scale pilot experiment. Prepare the ligations with different insert to vector ratios and transform the ligated product into JS-5 cells as described below. Use the insert:vector ratio that produces the highest number of recombinant plaques for library construction.
2. Extract the ligation reaction twice with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1). DNA stays in the top layer. Precipitate the extracted DNA with 0.1 volume of 3 *M* sodium acetate (pH 5.2) and 2.5 volume of ice-cold 100% ethanol. Pellet DNA by centrifugation at 14,000 rpm for 20 min. Discard ethanol and wash the pellet with 0.5 ml ice-cold 80% ethanol. Air dry the pellet and dissolve the DNA in 200 μl TE buffer.

The DNA can be stored at −80°C or taken directly to the transformation steps.

Transformation of Ligated DNA into Bacteria to Produce Phage Libraries

Electroporation competent JS-5 cells can be purchased from Stratagene (http://www.stratagene.com) or Bio-Rad (http://www.biorad.com), or prepared following the protocol described in Current Protocols in Molecular Biology.[13]

1. Aliquot 100 μl/tube of electroporation competent JS-5 cells into 50 pre-chilled microcentrifuge tubes.
2. Add 4 μl of ligated DNA to each tube.
3. Transfer the contents of each tube into a pre-chilled electroporation cuvette (0.2 cm pathlength). Use a kimwipe to remove any condensation on the outside of the cuvette to be electroporated. Place it in the electroporation chamber and electroporate ($V=2.0$ kV, $C=25$ μF, $R=400$ W).
4. Immediately add 1 ml of SOC to the cells.
5. Split each electroporation into two 6-ml Falcon tubes. Add 3 ml of 42°C Top Agar to each tube and immediately pour the entire contents onto a pre-warmed (37°C), 10-cm 2xYT plate. Incubate the plates first at room temperature for 10 min, allowing the top agar to solidify. Transfer and incubate the plates upside down in a 37°C incubator for 8 hr.
6. Elute the phage by adding 5 ml sterile PBS to each plate with gentle rocking at 4°C for 2–4 hr.
7. Collect the PBS (containing eluted phage) from each plate, combine them and centrifuge at 6000 rpm at 4°C for 10 min to spin out bacterial debris.
8. Transfer the supernatant to a clean tube, add 0.2 volume of 30% PEG 8000–1.6 *M* NaCl. Mix well. Incubate at 4°C for 1 hr to precipitate phage.
9. Pellet the phage by centrifugation at $10{,}000 \times g$ for 20 min at 4°C. Discard supernatant and centrifuge again to remove residual supernatant.
10. Resuspend phage pellet in 20 ml PBS–20% glycerol. Centrifuge at $6000 \times g$ for 10 min to remove any insoluble debris. Transfer the supernatant to a clean tube.
11. Dispense the phage library into 100–500 μl aliquots. Flash freeze in liquid nitrogen and store at −80°C.

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Affinity Selection of Nuclear Receptor Binding Peptides

Selection of peptides that bind to the target proteins of interest is achieved by incubating phage libraries containing small random peptide inserts with the target proteins that are immobilized on a solid support. Ninety-six-well microtiter plates are the most commonly used solid support for target immobilization. While paramagnetic beads, sepharose, immunotubes and other solid supports have been used successfully in phage display, 96-well microtiter plates afford easy handling of a large number of samples; thus they have always been the first choice in our laboratory. Most target proteins can be immobilized on the solid support using a basic solution ($NaHCO_3$, pH 8.5 or Tris–HCl, pH 8.5). The high pH promotes hydrophobic interactions between the target protein and the plastic. A very good alternative to immobilizing steroid–nuclear hormone receptors and other DNA-binding transcription factors is by using biotinylated oligonucleotides (corresponding to their cognate response element) pre-captured on streptavidin-coated plates. We will discuss both direct coating (using a basic solution) and DNA-mediated coating of target proteins in this chapter.

Materials

Anti-M13 antibody conjugated with horseradish peroxidase (Pharmacia)
Streptavidin (Sigma-Aldrich)
Adhesive lid (USA Scientific)
ABTS (2′-2′-azino-bis-ethylbenzthiazoline-6-sulfonic acid) solution.
Dissolve 10.5 g citric acid monohydrate in 1.0 liter sterile deionized water. Adjust pH to 4.0 with approximately 6 ml of 10 *M* NaOH. Add 220 mg ABTS.
Filter sterilize and store at 4°C, protecting from light.
Immediately before use, add 30% H_2O_2 to 0.05%.
Stock of TG-1 or DH5αF′ (Promega, Life Technologies, or Stratagene)
40% glycerol
Dilute 4 ml of glycerol with 6 ml deionized water.
Sterilize by autoclaving.
0.1 *M* HCl
Dilute 1 ml of concentrated HCl in 99 ml deionized water.
Sterilize by filtration.
1 *M* Tris–HCl, pH 7.4
Dissolve 74.5 g of Tris base in 800 ml H_2O.
Adjust the pH to 7.4 with HCl. Bring the final volume to 1000 ml with H_2O.
Sterilize by autoclaving.

2% Isopropylthio-β-D-galactoside (IPTG)
Dilute 20 mg of IPTG with 1 ml of H_2O.
Filter sterilize.
Store at −20°C.

100 m*M* $NaHCO_3$, pH 8.5
Dissolve 4.2 g of $NaHCO_3$ in deionized water to ~400 ml.
Adjust the pH to 8.5 if necessary.
Adjust the volume to 500 ml.
Filter sterilize, store at room temperature. Good for ~2 weeks.

1% BSA stock
Dissolve 100 mg of BSA in 10 ml deionized water.
Filter sterilize.
Dispense 1 ml aliquots into sterile microcentrifuge tubes.
Store at −20°C.

2% X-Gal, (5-bromo-4-chloro-3-indoyl-β-D-galactoside)
Dissolve 20 mg of X-Gal in 1 ml of dimethylformamide in glass or polypropylene tube.
Store wrapped in foil at −20°C.

Methods

Immobilization of Targets onto High-Binding Microtiter Plates

Method 1: Direct Adsorption of Proteins to the Plastic Wells.

1. Prepare the protein solutions to be used for immobilization by diluting your protein in 100 m*M* $NaHCO_3$ (pH 8.5) to a final concentration of 2.5 μg/ml immediately before use. Also prepare 100 μl of 2.5 μg/ml positive control protein solution in the same buffer.

   Note. The quality of the target protein is very important for a successful screen. Before the screen, steps need to be taken to make sure that the target proteins are not denatured during the purification steps or immobilization process, and that they are functionally active. Protein targets should be as pure as possible, free of any contaminants and affinity tags (6-His, GST, thioredoxin, etc.). Because of the strong selection power, many have noticed that if target proteins contain an affinity tag, peptides that bind to the affinity tag will be co-selected in the panning process, despite attempts to pre-block phage with reagents containing the affinity tag (see Ref. 17 and our own observation). If using an affinity tag is

[17] K. K. Murthy, I. Ekiel, S. H. Shen, and D. Banville, *Biotechniques* **26**, 142 (1999).

unavoidable, extra efforts will be required to weed out the tag binders from the target protein binding phage.

2. Obtain an Immulon 4 or equivalent high-binding microtiter plate. Mask every other row using narrow lab tape. It is important to skip wells in order to prevent cross contamination. Also, label one position to be used for the positive control. We frequently use estradiol-activated ERβ (Panvera, http://www.panvera.com) as a positive control in our screens.
   Note. Immulon 4 (Dynateck) plates provide higher protein-binding affinity. We have found, however, that certain phage appear to have high background binding to this plastic despite pre-blocking the wells with BSA and milk. We found that the Costar 96-well cell culture plates can work just as well for protein immobilization and appear to have the least background phage binding.
3. Add 100 μl of your target protein solution to each well to be used in panning. Add 100 μl of the positive control protein solution to the control well.
4. Seal the plate with an adhesive lid to avoid evaporation, and incubate the plate overnight at 4°C or room temperature for 2 hr.
5. After coating wells with target protein, add 150 μl of BSA (0.1% in PBS) or milk (nonfat dry milk, 2% in PBS) to each well to block nonspecific binding. Let incubate at room temperature for 1 hr. Wash wells five times with 300 μl of PBST (PBS + 0.1% Tween-20). The plate is now ready for panning.

Method 2: Tethering Target Protein to the Wells using Biotinylated Oligonucleotide. Transcription factors can bind to and form stable complexes with their DNA response elements, thus this class of proteins can be immobilized on a solid support through binding to oligonucleotide-coated wells. Synthetic biotinylated double-stranded DNA containing the response element is first immobilized on a streptavidin-coated plate (through a biotin–streptavidin interaction), then used to capture the target protein. Target protein immobilized using this method, presumably, resembles more closely the conformation it adopts in cells. We have also found in the ELISA assay that the conformation of ER is slightly different when bound to DNA. DNA bound ER allows more efficient recruitment of corepressor-like peptides than ER immobilized directly on the plastic.[18]

1. Prepare 10 μg/ml streptavidin in 100 m*M* $NaHCO_3$, pH 8.5 immediately before use.

[18] H.-J. Huang, J. D. Norris, and D. P. McDonnell, *Mol. Endocrinol.* **16**, 1778 (2002).

2. Add streptavidin solution to 96-well microtiter plates, seal the plate with an adhesive lid and incubate overnight at 4°C.
3. Block nonspecific binding sites with 150 μl of 2% milk (in PBS) or 0.1% BSA (in PBS). Incubate at room temperature for 1 hr.
4. Wash plate five times with 300 μl of PBST (PBS + 0.1% Tween-20).
5. Add 2 pmol of biotinylated double-stranded DNA (containing appropriate binding sequence for your target protein) in 100 μl of PBST to the wells. Incubate at room temperature for 1 hr.
6. Wash wells five times with 300 μl of PBST.
7. Add 2 pmol/well of your target protein (or 4 pmol, if your target protein binds as a dimer) diluted in 100 μl of PBST to the wells. Incubate at room temperature for 1 hr.
8. After incubation, wash wells five times with 300 μl of PBST. The plate is now ready for panning.

Affinity Partitioning of Binding Phage

In this step, phage containing random peptide libraries are incubated with the immobilized target. Nonspecific binding phage are removed by multiple washes and target-binding phage recovered by eluting with low pH buffer. The eluted phage are amplified in *E. coli* containing the F conjugative plasmid (F pili is required for M13 phage infection), such as DH5αF′ or TG-1. This process is repeated several times to enrich for target-binding peptides.

1. Start an overnight culture of DH5αF′ in 2xYT media from a single colony the day before panning. On the day of panning, dilute the overnight culture 1:100 into sufficient volume of 2xYT media and grow to log phase (OD = 0.5–1.0) to amplify the eluted phage from the day's panning.
2. Remove the blocking solution from target-coated wells by flicking the solution into the sink and "slapping" the inverted plate onto a stack of dry paper towels. Wash wells five times with 300 μl of PBST. Do not allow the wells to dry out completely.
3. Add 25 μl of random peptide library phage ($> 10^{10}$ pfu) in 125 μl of PBST to the corresponding labeled well. Seal the plates and incubate at room temperature for 2 hr.

   Note. We have found that pre-blocking the phage libraries with milk or BSA before applying them to the targets significantly reduces background binding phage in the panning process. Simply add milk (2%) or BSA (0.1%) to the phage aliquots in a microcentrifuge tube and incubate on ice for 1 hr before applying them to the target wells.

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Note. To avoid contamination, frequent changes of gloves is recommended. Also, aerosol resistant pipet tips should be used for all pipetting involving phage.

4. Remove nonbinding phage with five washes of PBST, then another five washes of PBS.
5. Elute the bound phage by adding 100 μl of 0.1 *M* HCl to the well and incubate for 10 min at room temperature. Neutralizing the eluted phage immediately with 50 μl of 1 *M* Tris–HCl (pH 7.4) and proceed directly to amplification, or store at 4°C for up to 2 weeks.
6. Save 25–50 μl of eluted phage to determine the elution titer. Mix 5 ml of log-phase DH5αF′ (or TG-1) with the rest of the eluted phage. Incubate first in 37°C water bath for 20 min without shaking, then in a 37°C shaking incubator for 3–8 hr.
   Note. The amplification is ideally for no longer than 8 hr to minimize the chance of proteolytic degradation of displayed peptides.
   Depending on the titer of the eluted phage, typically a 5-hr amplification should be sufficient.
7. Spin down cells at $4000 \times g$ for 10 min, then transfer the supernatant (containing amplified phage) to a new tube. Heat at 65°C for 2 min to pasteurize the supernatant. Store at 4°C until use. Also make aliquots of frozen stock for long-term storage and for archival purposes in case there is a need to re-pan or analyze the isolates from this selection (mix 1 volume of phage supernatant with 1 volume of sterile 40% glycerol, flash freeze in liquid nitrogen, and store at −80°C). Titer the amplified phage as described below.
8. Prepare target-coated plates as described above and repeat the affinity selection using 10^9–10^{12} pfu of the amplified phage. Repeat this process 2–3 times to enrich for target-binding phage. An increase in the elution titers following subsequent rounds of panning is an indication of enrichment. The panning stringency can be increased by lowering the input phage to as low as 10^9 pfu, or by decreasing the target protein coated on the well.

Determine Phage Titer

1. Pre-warm a sufficient number of 2xYT plates in 37°C incubator.
2. Make serial 10-fold dilutions of phage supernatant in PBST.
3. Mix 30 μl 2% X-Gal, 30 μl 2% IPTG, 200 μl log-phase DH5αF′ and 100 μl phage dilutions in a 10 ml tube. Incubate for 20 min at 37°C without shaking.

4. Add 3 ml of melted top agar (kept at 50°C) to each tube, mix well and spread the contents evenly on a 2xYT plate. Incubate the plates at 37°C for 8 hr to overnight until blue plaques are visible and can be counted.

Determination of Binding Activities within Phage Pools by ELISA

1. Coat target proteins on microtiter plates for ELISA the same way as for panning except that one well of the target must be prepared for each round of selection for each library that was panned. Typically, pool ELISAs are performed after 3–4 rounds of panning. Include an appropriate number of wells for the positive and negative controls.
2. Block and wash the wells as before.
3. Add 50 μl of PBST to each well containing immobilized protein to prevent wells from drying out, then add an equal number of phage (10^9–10^{12} pfu/well) from the phage stock representing each pool to the appropriate wells. Seal the plates and incubate at room temperature for 1 hr.
4. Remove nonbinding phage by washing the wells five times with PBST.
5. Dilute horseradish peroxidase-conjugated anti-phage antibody 1:5000 in PBST. Add 100 μl of the diluted antibody to each well. Seal the wells and incubate the plate at room temperature for 1 hr. Wash the wells five times with PBST.
6. Add 100 μl ABTS reagent containing 0.05% H_2O_2 to each well. Incubate the plate at room temperature for 10 min. Measure the absorbance at 405 nm with a microtiter plate reader.
   Note. The ELISA signal from pools of a particular library should show a steady increase for subsequent rounds (Fig. 6). If enrichment is not observed after four rounds, then there is a good chance that the target is denatured or inactive. Consider an alternative method of immobilization.

Isolation and Propagation of Affinity-Purified Phage Clones

1. Melt top agar in a microwave and keep it at 50°C until ready for use. Pre-warm 2xYT plates in a 37°C incubator. Plan ahead to make sure that a fresh log-phase culture of an appropriate host strain (DH5αF′ or TG-1) is ready for use.
2. In a 96-well plate, perform serial 10-fold dilutions of phage stocks which gave best results in the ELISA assay.

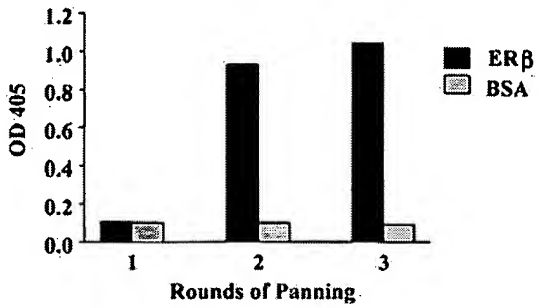

FIG. 6. Enrichment of target binding phage during panning process. A CoRNR box library in the format of [X7-LXX(H/I)IXXX(I/L)-X7] corresponding to the receptor interacting domain of the NCoR and SMRT corepressors was constructed and used to screen for peptides that bind ERβ in the presence of ICI 182,780.[18] To prepare the target for these screens, a biotinylated ERE was first immobilized on a 96-well microtiter plate precoated with streptavidin. ERβ was then immobilized on the coated EREs in the presence of ICI 182,780 (1 μM). Panning was performed by incubating 10^{10} pfu of library with the target overnight at 4°C. After washing, the bound phage were eluted, amplified and subjected to two additional rounds of panning. Enrichment of ERβ specific binding phage was assessed by measuring the amount of phage bound to ERβ-coated versus BSA-coated wells using an anti-phage ELISA.

3. Mix 30 μl 2% X-Gal, 30 μl 2% IPTG, 200 μl log-phase DH5αF′ and 100 μl phage dilution in a 10 ml tube. Incubate for 20 min at 37°C without shaking.
4. Add 3 ml of melted top agar to each tube, mix well, and spread the contents evenly on a 2xYT plate. Allow the plates to sit undisturbed for 10 min until the top agar hardens. Incubate the plates at 37°C for 8 hr to overnight, until the blue plaques are easily identifiable. By this time, some of the dilutions should have produced isolated single plaques. Store plates at 4°C until ready to pick up the isolated phage clones.
5. For each isolated plaque to be propagated, add 3 ml of log-phase DH5αF′ to a 15 ml tube. At least 48 plaques should be picked from each pool. Amplification can be done in 96-well, deep well plates (2 ml volume). Grow a 1-ml culture for each individual plaque isolated.
6. Pick (touch and twist) and inoculate a blue isolated plaque into each of the 15-ml tubes (or 96-well deep well plates). Incubate at 37°C with vigorous agitation for 6 hr.
7. Pellet the bacterial cells by centrifugation at 4°C, 4000 $\times$ g for 10 min. Transfer phage supernatant into a new tube and store at 4°C until ready to use. The bacterial pellet can be used for plasmid preparation for sequencing.

Confirmation of Binding Activity of Individual Phage Clones by ELISA

1. Coat a microtiter plate with target protein for ELISA as described above. For each phage clone to be tested, prepare one well coated with target protein and another well coated with a negative control protein, such as the bacterial fusion partner (i.e., GST, maltose-binding protein, etc.), milk or BSA. It is not necessary to skip wells at this time. An appropriate positive control for the ELISA should also be included.
2. Block and wash the wells as described before.
3. Add 50 μl of PBST to each well containing immobilized protein to prevent wells from drying out. Add 50 μl of each phage supernatant to be tested to each set (target and negative controls) of wells. Seal the plates and incubate at room temperature for 1 hr.
4. Wash the wells five times with PBST.
5. Dilute horseradish peroxidase-conjugated anti-phage antibody 1:5000 in PBST. Add 100 μl of the diluted antibody to each well. Seal the wells and incubate the plate at room temperature for 1 hr. Wash the wells five times with PBST.
6. Add 100 μl ABTS reagent containing 0.05% H_2O_2 to each well. Incubate at room temperature for 10 min. Quantify the reaction by measuring the absorbance at 405 nm with a microtiter plate reader. Optical density (OD) values in the range of 0.5–3.0 generally constitute positive signals, while negative signals are typically in the range of 0.05–0.3, although your individual experience may differ.
7. Re-plaque and re-ELISA the positive phage prior to subsequent analysis. Only those isolates whose activity is confirmed by ELISA should be carried forward for subsequent analysis (sequencing and mammalian two-hybrid assays).

Validation of Receptor-Interacting Peptides in Mammalian Cells

Although an *in vitro* ELISA assay can verify the interaction between the isolated peptide and the target receptor, a secondary approach is often required to eliminate peptides that may be binding to a minor contaminant in the protein preparation or to the fusion tag. There is also concern that the purified receptor, produced either in bacterial or insect cells, may not have the same conformation and/or post-translational modification(s) as the proteins expressed in mammalian cells. We have therefore implemented

a cell-based assay to further validate the peptides obtained from phage display screens.

The cell-based assay is a modification of a commonly used mammalian two-hybrid analysis. Isolated peptides are made as fusion proteins to the Gal4-DBD and the receptor of interest is made as a fusion partner of the VP16 acidic transactivation domain. The assay consists of co-transfection of the Gal4DBD-peptide and VP16-receptor plasmids into mammalian cells together with a luciferase reporter gene containing a Gal4 response element. If the DNA-bound peptide can interact with the receptor in target cells, the VP16 activation domain, via its fusion to the receptor, will be brought to the DNA and enable the expression of the reporter gene. An added advantage of moving the validation step into mammalian cells is the ability to cross screen multiple nuclear receptors without having to purify individual receptors for *in vitro* assays. We have used this approach to test the receptor-binding specificity of phage identified using ERα and ERβ as bait in our primary screens. With ease we evaluated the ability of over 50 peptides to interact with 10 different receptors using this approach.[19,20] ER subtype-specific peptides were identified and have proven to be very useful in dissecting ERα and ERβ signaling. Non-discriminating peptides that bind multiple receptors have also been useful for studying the mechanism of action of those receptors. For example, peptides identified in ERα screens have been used to study the mechanisms of action of the androgen receptor (AR),[21] retinoic receptor-related orphan receptor-alpha (RORα),[22] and the Vitamin D3 receptor (VDR)[23] without having to initiate a primary screen against these receptors.

Materials

pM and pVP16 vectors can be obtained from Clontech (http://www.clontech.com).

pM-peptide and pVP16-receptor constructs are made using regular cloning techniques.

Lipofectin, cell culture media (Life Technologies).

[19] C.-Y. Chang, J. D. Norris, H. Grøn, L. A. Paige, P. T. Hamilton, D. J. Kenan, D. Fowlkes, and D. P. McDonnell, *Mol. Cell. Biol.* **19**, 8226 (1999).

[20] J. M. Hall, C.-Y. Chang, and D. P. McDonnell, *Mol. Endocrinol.* **14**, 2010 (2000).

[21] C.-Y. Chang and D. P. McDonnell, *Mol. Endocrinol.* **16**, 647 (2002).

[22] C. D. Kane and A. R. Means, *EMBO J.* **19**, 691 (2000).

[23] P. Pathrose, O. Y. Barmina, C.-Y. Chang, D. P. McDonnell, N. K. Shevde, and J. W. Pike, *J. Min. Bone Res.* **17**, 2196 (2002).

Method

1. Human hepatocarcinoma cells HepG2 are split into 24-well plates the day before transfection. We have performed this assay in a number of other cell lines with similar success.
2. For triplicate wells using lipofectin-mediated transfection, we use
   400 ng p*M*-peptide plasmid
   400 ng pVP16-receptor plasmid
   200 ng pCMV-βgal
   2000 ng 5 × Gal4-Luc3 reporter plasmid
   The amounts of DNA used vary with different transfection media. One can modify the input DNA amounts to accommodate the requirements of that particular transfection protocol.
3. Perform transfection using your chosen transfection protocol.
4. After transfections, add the appropriate hormone to the cells if the receptor–peptide interaction is expected to be ligand-dependent. Incubate the cells for 24 hr. Perform luciferase and β-galactosidase assays according to manufacturer's instruction.

Use Receptor-Interacting Peptides to Inhibit Receptor Transcriptional Activity

Since cofactor–receptor interactions are required for nuclear receptors to fully manifest transcriptional activity, disruption of such interactions is expected to have an inhibitory effect on receptor activity. Many of the peptides identified in the phage display screens have been demonstrated to bind to important protein–protein interaction surfaces on the receptors. For example, several of the peptides we identified contain an LxxLL motif that mimics the receptor interaction domain(s) of the p160 class of coactivators.[9,19] Two major concerns that may limit the use of these peptides as antagonists of nuclear receptors are: (1) the selected peptides may not possess high enough affinity to disrupt receptor–cofactor interactions, and (2) the peptides obtained may bind to multiple nuclear receptors. To address the first concern, we have used fluorescence polarization assays to measure the affinity of some of our ER-binding peptides and found that most bind in the 100 n*M* range. One of the peptides identified in our primary screen, however, possesses an affinity of 60 n*M* for ERβ, similar to the affinity of coactivator SRC-1 to ERα.[24,25] Not surprisingly, when introduced into cells,

[24] M. Jansen, personal communication.

[25] E. Margeat, N. Poujol, A. Boulahtouf, Y. Chen, J. D. Muller, E. Gratton, V. Cavailles, and C. A. Royer, *J. Mol. Biol.* **306**, 433 (2001).

these peptides efficiently blocked ERα and ERβ transcriptional activity.[19,20] In addition, peptides that demonstrate receptor-specific binding characteristics have also been identified, eliminating our second concern. We found two peptides which bind specifically to ERβ but show no interaction with any other nuclear receptors tested.[20] These peptides are powerful tools to dissect the pharmacology of the closely related ERα and ERβ isoforms, where no ER subtype-specific ligands are available. We believe that the same approach can be applied to other nuclear receptors, particularly the orphan nuclear receptors where there is no known ligand to modulate their activity.

The simplest way of introducing these peptides into mammalian cells is to transfect plasmids encoding the peptide (or peptide fusion proteins, i.e., Gal4-DBD fusion used in the mammalian two-hybrid assay) using transient transfection. We have also had success expressing peptides in cells and in whole animals using an adenoviral delivery system. Furthermore, we have found stable cell lines expressing peptides under the control of a regulated promoter to be useful in our studies. Also available are several emerging technologies which allow the introduction of synthetic peptides or *in vitro* purified recombinant protein–peptides into cells.[26–32]

In conclusion, we have described in this chapter the use of a combinatorial peptide approach to dissect the cofactor–nuclear receptor interface. We have also discussed the use of these peptides in the study of nuclear receptor pharmacology. We believe that the same approach can be applied to other cellular proteins whose activity is modulated by protein–protein interactions. For example, phage display has been used to identify peptides which disrupt the interaction between htm2 and p53, preventing htm2-mediated p53 degradation, leading to subsequent stabilization and activation of p53 in cells.[33,34] The full potential of using phage display to study protein–protein interactions we believe has yet to be realized. We hope that the examples presented here, where it has been used to study

[26] E. Vives, P. Brodin, and B. Lebleu, *J. Biol. Chem.* **272**, 16010 (1997).
[27] S. Fawell, J. Seery, Y. Daikh, C. Moore, L. L. Chen, B. Pepinsky, and J. Barsoum, *Proc. Natl. Acad. Sci. USA* **91**, 664 (1994).
[28] S. R. Schwarze, A. Ho, A. Vocero-Akbani, and S. F. Dowdy, *Science* **285**, 1569 (1999).
[29] D. Derossi, A. H. Joliot, G. Chassaing, and A. Prochiantz, *J. Biol. Chem.* **269**, 10444 (1994).
[30] G. Elliott and P. O'Hare, *Cell* **88**, 223 (1997).
[31] P. A. Wender, D. J. Mitchell, K. Pattabiraman, E. T. Pelkey, L. Steinman, and J. B. Rothbard, *Proc. Natl. Acad. Sci. USA* **97**, 13003 (2000).
[32] D. J. Mitchell, D. T. Kim, L. Steinman, C. G. Fathman, and J. B. Rothbard, *J. Pept. Res.* **56**, 318 (2000).
[33] A. Bottger, V. Bottger, A. Sparks, W. L. Liu, S. F. Howard, and D. P. Lane, *Curr. Biol.* **7**, 860 (1997).
[34] V. Bottger, A. Bottger, S. F. Howard, S. M. Picksley, P. Chene, C. Garcia-Echeverria, H. K. Hochkeppel, and D. P. Lane, *Oncogene* **13**, 2141 (1996).

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nuclear receptor function, will encourage other investigators to utilize this technology.

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